Chromosome doubling in *Tripsacum*: the production of artificial, sexual tetraploid plants

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Abstract

A collection of embryogenic diploid calli of *Tripsacum* was established and treated with colchicine to induce chromosome doubling. Sections containing duplicated cells in calli were identified using flow cytometry and ploidy level was determined in the regenerated plantlets. Tetraploid plants from several origins were obtained. In contrast to wild polyploid plants, which show apomictic development, the regenerated tetraploid plants reproduced sexually. By hybridizing these plants with wild tetraploid apomicts, various populations were established; these will allow a study of the inheritance of apomixis in *Tripsacum*.

Key words: *Tripsacum* — apomixis — callus culture — chromosome doubling — colchicine — flow cytometry — sexual tetraploid

Gametophytic apomixis, that is, asexual reproduction through seeds (Nogler 1984), could greatly improve grain-crop production in the tropics and simplify breeding if transferred to major crops (Bashaw 1980, Savidan and Dujardin 1992). The ORSTOM-CIMMYT *Tripsacum* project is attempting the transfer of apomixis into maize from *Tripsacum*, maize's closest apomictic wild relative, via the conventional backcross pathway (Savidan and Berthaud 1994). Simple inheritance is one prerequisite for transferring genes of interest through wide crosses from wild relatives into crops. Apomixis results from two basic events: non-reduction (i.e. apospory or diplospory) followed by egg-cell parthenogenesis, which have been reported to be genetically linked (Savidan 1982, Nogler 1984). Studies in a few tropical forage grasses and dicotyledons have clearly demonstrated that apospory has a monogenic inheritance (Nogler 1984, Asker and Jerling 1992), but the inheritance of diplospory has still to be elucidated.

The mode of reproduction in species of the *Tripsacum* agamic complex is closely related to ploidy level. Diploid accessions ($2n = 36$) are sexual, whereas the polyploids ($2n = 54, 72, 90$ and 108) reproduce through diplosporous, gametophytic apomixis (Leblanc et al. 1995). For genetic analyses of the inheritance of apomixis, apomixis and sexuality must be expressed at the same ploidy level to avoid meiotic disturbances or sterility; these may result from unstable meiotic ploidy levels (e.g. a triploid resulting from diploid × tetraploid crosses) in populations that segregate for the mode of reproduction. Because apomixis is commonly expressed at the tetraploid level in the wild and because apomictic polyhaploid plants have been gen-

erally described as weak and sterile (Nogler 1984), the production of artificial sexual tetraploid plants through colchicine chromosome doubling of diploid calli was attempted. Flow cytometry, allowing easy and quick estimation of chromosome number in plant tissues (Galbraith et al. 1983, Delaat et al. 1987), was used to estimate DNA content in untreated and treated calli and in regenerated plantlets.

Materials and Methods

Establishing diploid callus cultures: Calli were induced using mature embryos obtained from open-pollinated diploid accessions and from controlled crosses between diploid accessions from the ORSTOM-CIMMYT collection maintained at the CIMMYT experiment station at Tlaltizapán, Morelos State, Mexico. For callus induction, 45 mature embryos were carefully removed from sterilized seeds and transferred to flasks containing 30 ml of agar-solidified N_6 medium (Chu et al. 1975) supplemented with dicamba (2 mg/l). After 3 weeks in the dark at 26°C, embryo-like structures were removed and subcultured for multiplication on the same callus-induction medium in the dark at 26°C at 3-week intervals. The ploidy and regeneration capacity of the calli were checked after 6 cycles of subculture, the former using flow cytometry.

Colchicine treatment: A stock solution of colchicine was filter-sterilized and added to the autoclaved agar N_6-medium before solidification. Three levels of colchicine treatment, 0.05, 0.1 and 0.2%, were applied for each selected callus line. Embryogenic calli were divided into small pieces (0.5–1 mm²) and the pieces were placed in flasks containing 30 ml of the agar-solidified N_6 medium supplemented with colchicine. After 72 h of incubation at 26°C in the dark, the calli were removed and subcultured twice (at 1-week and 3-week intervals) on N_6 medium supplemented with dicamba (2 mg/l), before checking ploidy levels. Each callus was divided into 2–4 sections and each section was analysed using flow cytometry. Sections that showed signs of chromosome doubling were subcultured three times (at 3-week intervals) before regeneration.

Plant regeneration: Regeneration was initiated by transferring calli to a medium containing MS salts (Murashige and Skoog 1962) supplemented with 20 g/l of sucrose, 0.15 g/l of L-asparagine, 0.5 mg/l of IAA (indol 3-acetic acid), 1 mg/l of BAP (6-benzylaminopurine), and 0.4 mg/l of thiamine. The medium was then adjusted to pH 5.7 with 1M NaOH. Calli were incubated with 18 h light per day at 26°C. Once leaf shoots developed (5–10 cm), they were transferred to a rooting medium containing MS salts supplemented with 20 g/l of sucrose and 1 mg/l of NAA (α-naphthaleneacetic acid). After 10–15 days, plantlets
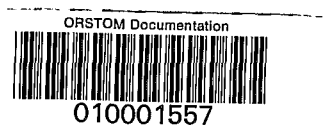
were transplanted to soil in pots and transferred to the greenhouse. Finally, each regenerated plant was analysed for ploidy level using flow cytometry, before transfer to the field. In addition, root tips from 5–10 tetraploid regenerated plants per duplicated callus line were prefixed at 4°C in saturated 8-hydroxyquinoline for 4 h, transferred to fresh Carnoy's solution (6:3:1, v/v/v, 95% ethanol:chloroform:acetic acid), fixed for 24 h and stored at −4°C in 70% ethanol. Chromosome counts, to confirm flow cytometry analyses, were made on metaphase cells from squashed root tips and observed by phase-contrast microscopy.

Flow cytometry: A Partec CA II cytometer equipped with the DAPI staining filter combination and with DPAC software for data analyses (Partec GmbH, Münster, Germany) was used for DNA-content estimations. Small pieces of both calli and leaves were chopped using a razor blade in 10 ml of the chopping buffer, as described by Galbraith et al. (1983), containing 2 μg/ml of bis-benzimidine, a fluorochrome that binds to DNA. The solution was then passed through a nylon filter (pore size, 40 μm) and the stained nuclei in the filtrate analysed by flow cytometry. The fluorescence measured is correlated with the DNA content of the stained nuclei. For each peak detected, an index can be calculated using a given reference (e.g. a peak corresponding to a cell mixture of known DNA content); therefore the comparison of the index gives the relationship between the DNA contents in the stained nuclei that produced those peaks.

Prior to carrying out experiments with treated calli and regenerated plantlets, the DNA contents of maize CIMMYT line CML 62 and diploid *Tripsacum* (untreated calli and leaves) were estimated to determine whether this line could be used as an internal control by chopping its leaves together with the *Tripsacum* samples.

Determining the mode of reproduction: In species that produce mono- and bisporic embryo sacs, sexual megasporogenesis is characterized by callose deposition in megasporocyte and megaspore cell walls (Rodkiewicz 1970). In the *Tripsacum* agamic complex, callose is also critical for meiosis, but diplosporous megasporocytes lack callose deposition throughout megasporogenesis (Leblanc et al. 1995). For callose analysis, pistils were dissected from young female parts of inflorescences, optically cleared and stained using three sucrose solutions (250, 500, and 800 g/l successively), supplemented with aniline blue (100 mg/l), and finally examined using ultraviolet vertical epifluorescence microscopy according to Leblanc et al. (1995). Ovules from 10–25 flowers from each regenerated tetraploid genotype were analysed.

Results

The DNA contents of the maize line and the diploid *Tripsacum* samples, either from leaf tissue or callus, were similar when estimated using flow cytometry (Fig. 1a, b). However, peaks from maize and *Tripsacum* did not generally overlap perfectly; coefficients of variation (CV) were around 5% for *Tripsacum* and maize-cell mixtures, but only 3% in pure maize-cell mixtures (Fig. 1c). Therefore, a peak at about twice the index of the peak corresponding to the maize cells was expected in cases of chromosome doubling.

From the 45 mature embryos used for callus induction, 31 callus cultures were established of which 26 were diploid (Table 1). Although tissue culture may induce endoreplication, the polyploid cultures recovered may also come from fertilization of a reduced female gamete by 2x pollen (tetraploid parent), or from fertilization of an unreduced female gamete by 1x or 2x pollen. Among the 26 diploid cultures previously identified, 17 were found to be highly regenerative and were selected for chromosome-doubling experiments (Table 1). All the regenerated plants from the selected calli were diploid, as determined by flow cytometry (Fig. 1a).

There were two principal signs of chromosome doubling in calli when cell mixtures were prepared from maize and treated calli tissues and analysed using flow cytometry. The first was a high percentage of tetraploid cells (more than 20%). In that case, the peak corresponding to the tetraploid cells in the mixture (peak 2, Fig. 2) represented maize cells or diploid cells from the callus in phase G2 of the cell cycle (about 15–20%, peak 2, Fig. 1b), and *Tripsacum* tetraploid cells resulting from chromosome doubling. The second indicator of chromosome doubling was small peaks of 8x DNA content, representing *Tripsacum* tetraploid cells in phase G2 (peak 3, Fig. 2). Two subcultures after the colchicine treatment, six callus lines had one or more sections containing cells affected by the colchicine treatment. Three subcultures later, in all sections previously selected except two (from C-14 and C-62), such cells were detected in varying proportions (Table 2). When effective, colchicine treatment of diploid calli yielded tetraploid cells, except

	Index	Area %	CV%
peak 1	1.02	63.33	5.56
peak 2	1.96	17.35	4.80

	Index	Area %	CV%
peak 1	1.01	66.96	5.77
peak 2	1.99	18.67	4.91

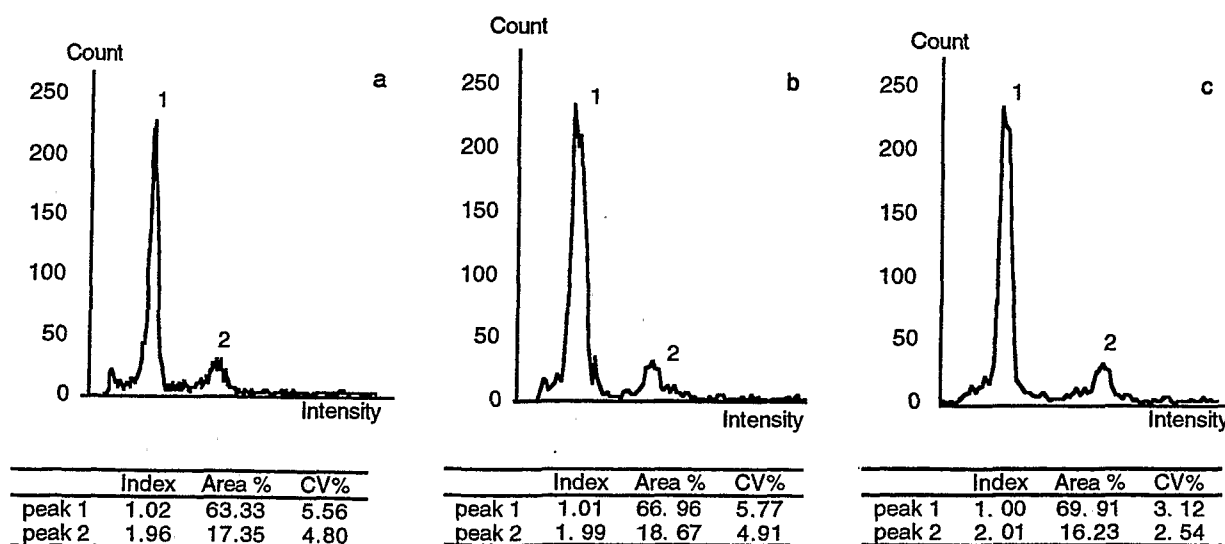|	Index	Area %	CV%
peak 1	1.00	69.91	3.12
peak 2	2.01	16.23	2.54

Fig. 1: Relative DNA contents in maize (CIMMYT line CML62) and *Tripsacum* estimated by flow cytometry: a. Cell mixture prepared from leaf tissues of both maize and a diploid regenerated plant from untreated callus line C-48; b. Cell mixture prepared from untreated callus line C-48 and leaf tissue from maize; c. Cell mixture of pure maize. Peak 1: diploid cells. Peak 2: diploid cells in G2

Table 1: Main characteristics of the induced *Tripsacum* calli. All the *Tripsacum* accessions, except those from population #111, seeds of which were collected *in situ*, are from populations belonging to collections maintained at the CIMMYT experiment station, Tlaltizapán, Morelos State, Mexico. Species designations are: DM: *T. dactyloides* var. *mexicanum*; DH: *T. dactyloides* var. *hispidum*; MR: *T. dactyloides* var. *meridionale*; MZ: *T. maizar*; PL: *T. pilosum*; ZP: T. zopilotense

Mature embryo origin		Induced callus ploidy level			Highly regenerative diploid lines
		$2n = 36$	$2n = 54$	$2n = 72$	
Open-pollinated accessions					
Tripsacum accessions	Species				
39–1569	PL	2	2	1	2
46–404	PL	2			1
51–470	ZP	1	1		1
population #111	DH	2			–
Controlled crosses					
Tripsacum accessions	Species				
39–1569 × 8–559	PL, MZ	2			1
39–310 × 47–410	PL, PL	3			2
47–410 × 49–1389	PL, ZP	1			1
75–911 × 39–1830	DH, PL	1			1
76–916 × 47–415	DM, PL	6	1		5
99–1099 × 575–5136	MZ, MR	3			1
7203–3 × 47–410	MZ, PL	2			2
575–5136 × 8–559	MR, MZ	1			–
Total		26	4	1	17

in one section from line C-48 (0.1%) in which a high proportion of octoploid cells was observed. However, when chromosome doubling was detected, the CV values of the 'control' peaks from mixtures prepared from treated calli were similar to those prepared from untreated calli (e.g. peak 1, Fig. 1b and Fig. 2). This indicated that all the treated calli showing chromosome doubling were certainly mixoploid. Altogether, 1109 plants from three of the six lines showing duplicated cells at the fifth subculture after treatment were regenerated (Table 2); 249 (22.5%) were tetraploid (Fig. 3), confirming the mixoploid nature of the calli affected by the colchicine treatment. Of these, 237 survived and were transferred to pots. No octoploid plants were obtained either from partial tetraploid calli or from the octoploid section from callus line C-48. Of the 249 tetraploid plants recovered, 11 (4.5%) were regenerated from callus treated with 0.05% colchicine. All others came from 0.2% colchicine-treated calli, suggesting that high concentrations of colchicine are required for mitotic arrests in *Tripsacum*. In contrast to the treated calli, analyses of leaves from regenerated plants showed lower CVs for the 'control' peak (e.g. around 3%, peak 1, Fig. 3) suggesting that the regenerated plants were tetraploid and not chimaeric. Root-tip counts confirmed the tetraploid nature of the plantlets selected using flow cytometry (5–10 for each genotype).

Callose fluorescence around megaspore mother cells and their derivatives was observed both in diploid plants regenerated from untreated calli and in tetraploid plants regenerated from colchicine-treated calli (Fig. 4). These observations are typical of sexual development in *Tripsacum* spp. (Leblanc et al. 1995), and clearly indicate sexual reproduction at the tetraploid level.

Discussion

Flow cytometry greatly increased the efficiency of screening ploidy levels at critical steps in the experiment — diploid callus

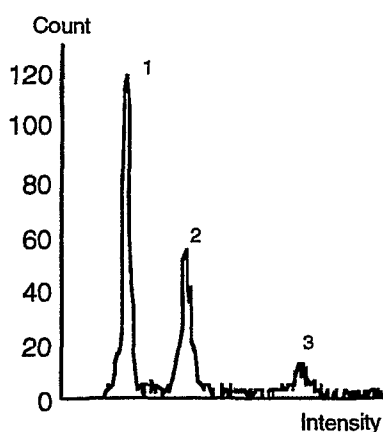|	Index	Area %	CV%
peak 1	1.00	42.91	5.00
peak 2	1.94	32.87	4.55
peak 3	3.76	8.99	2.66

Fig. 2: Estimation of the ploidy levels in one section of callus-line C-48 five subcultures after 0.2% colchicine treatment. Callus tissue was analysed together with CIMMYT maize-line CML62. Peak 1: diploid cells from both the maize and the callus. Peak 2: maize cells in G2 and tetraploid cells from callus. Peak 3: Tetraploid cells from callus in G2

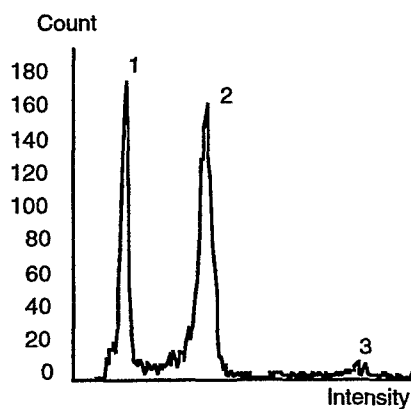|	Index	Area %	CV%
peak 1	1.23	30.74	3.19
peak 2	2.41	49.70	3.23
peak 3	4.58	1.99	1.69

Fig. 3: Estimation of the chromosome number in a tetraploid plant regenerated from colchicine-treated callus-line C-48. Leaf tissue was analysed together with CIMMYT maize-line CML62. Peak 1: maize cells. Peak 2: tetraploid cells from the regenerated plant. Peak 3: tetraploid cells from the regenerated plant in G2

Table 2: Levels of polyploidy in calli of *Tripsacum* spp. five subcultures after colchicine treatment (all of them showed signs of chromosome doubling two subcultures after treatment) and in their respective regenerated plants, as determined by flow cytometry. ND = not determined; NR = callus line survived but did not regenerate plantlets

#	Callus line embryo origin	Colchicine treatment	Polyploid levels detected (% of cells analysed)		Number of regenerated plants		
			4x	8x	Total	4x	8x
C-12	76–916 × 47–415	0.05%	24.50	traces	29	–	–
C-14	76–916 × 47–415	0.05%	19.10	–	197	11	–
		0.2%	15.02	–	26	–	–
C-48[1]	47–410 × 49–1389	0.1%	20.32	traces	NR	–	–
		0.1%	traces	44.64	NR	–	–
		0.2%	ND	ND	24	23	–
		0.2%	32.87	8.99	55	37	–
C-49[2]	75–911 × 39–1830	0.2%	39.15	4.23	248	107	–
		0.2%	42.26	6.48	173	48	–
		0.2%	45.23	traces	246	23	–
C-62	99–1099 × 575–5136	0.1%	37.13	6.56	84	–	–
		0.2%	15.00	–	27	–	–
Total regenerated plants					1109	249	–

[1] Two sectors showed duplications at 0.1 and 0.2% colchicine
[2] Three sectors showed duplications at 0.2% colchicine

Fig. 4: Callose fluorescence during megasporogenesis in a tetraploid regenerated plant: tetrad of megaspores with fluorescing cell walls (× 650)

selection, identification of polyploid sections in calli, and determination of ploidy level in regenerated plants. Conventional chromosome counting involves staining mitotic cells at metaphase, a time-consuming procedure that greatly limits the number of counts. Moreover, the effects of colchicine treatment can only be appreciated after plantlet regeneration. Using flow cytometry, ploidy is quickly estimated whatever the plant tissue and promising materials can be detected before regeneration and transferred into pots.

Sexuality in grass agamic complexes is restricted to the diploid gene pool and the interrelationship between polyploidy and gametophytic apomixis is strong (Stebbins 1950). Changes in mode of reproduction after chromosome doubling from colchicine treatment of sexual diploids related to polyploid apomicts has rarely been reported (*Calama grostis* Nygren 1948; *Paspalum*: Quarin and Hanna 1980), but, at least in *Paspalum*, low levels of apomixis expression (up to 5%) have been reported in several diploid species (Norrmann et al. 1989). By contrast, chromosome doubling in many sexual diploids belonging to agamic complexes does not affect the mode of reproduction

(Asker and Jerling 1992), as was the case for the *Tripsacum* materials whose chromosomes were duplicated. This may indicate that polyploidy alone is not sufficient to cause a breakdown in the sexual reproduction of autotetraploids recovered from diploids related to apomicts.

The sexual tetraploid *Tripsacum* plants reported here will be used as pistillate parents in crosses with tetraploid apomictic accessions that produce fertile reduced pollen. The resulting populations are expected to segregate for the mode of reproduction (sexuality vs. apomixis) allowing the genetic study of apomixis components (diplospory, parthenogenesis) in *Tripsacum*. The inheritance of gametophytic apomixis has generally been studied in aposporous materials. Although evidence of simple inheritance of diplospory has been reported in *Eragrostis curvula* (Voigt and Bashaw 1972), no comprehensive analysis of the inheritance of apomixis in diplosporous materials exists.

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